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(71) Applicant: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventor: CLAUSEN, Kim; Hovedgaden U 12, DK-4340 Tølløse (DK).

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(54) Title: AN ENZYMATIC OIL-DEGUMMING PROCESS

(57) Abstract

An improved process for enzymatic reducing the content of phosphorus containing components in an edible oil. The method comprises the use of phospholipase and a low amount of water.

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TITLE:

An enzymatic oil-degumming process

FIELD OF INVENTION

The present invention relates to an improved process for enzymatic reducing the content of phosphorus containing components in an edible oil.

BACKGROUND OF THE INVENTION

Oils obtained from the usual oil and fat production processes by compressing oil-bearing materials or by extracting oil from the materials and removing the extraction solvent contain impurities such as polar lipids mainly composed of phospholipids, as well as fatty acids, pigments, odor components and the like. Thus it is necessary to remove these impurities by a refining process. Such a process may require a degumming step.

In the art it is known to use phospholipase for enzymatic degumming of an edible oil (US 5,264,367; JP-A-2153997; and EP 622446), to reduce the phosphorus content of said water degummed edible oil.

However those references do not specifically suggest to use low amount of water in the enzymatic degumming process.

In contrary EP 622446 suggest to use high amount of water in the enzymatic degumming process. See page 3, line 33-44 and claim 4 in said document, which suggest to use more than 30 percent of water by weight of the oil in said process.

SUMMARY OF THE INVENTION

The problem, to be solved, by the present invention is to provide a simplified and economically cheaper process for enzymatic degumming of edible oils.

The solution is to perform said process using low amounts of water.

Accordingly, the present invention relates to a process for reducing the content of phosphorus containing components in an edible oil, having from 50 to 10.000 part per million (ppm) of phosphorous content, which method comprises contacting said oil at a pH from 1.5 to 8 with an aqueous solution of a

phospholipase A1 (PLA1), phospholipase A2 (PLA2), or phospholipase B (PLB) which is emulsified in the oil until the phosphorous content of the oil is reduced to less than 12 ppm, and then separating the aqueous phase from the treated oil, and wherein said process is characterized by that said emulsified condition is formed using from 0.01 to 1.5 percent of water by weight of the oil, preferably from 0.01 to 1.0 percent of water by weight of the oil, more preferably from 0.01 to 0.75 percent of water by weight of the oil, even more preferably from 0.01 to 0.5 percent of water by weight of the oil, and most preferably from 0.01 to 0.4 percent of water by weight of the oil.

Further, the lower range above of 0.01 percent of water by weight of the oil, may preferably be 0.1 percent of water by weight of the oil.

An advantage of the process described herein is that costs for water and waste water treatment may be reduced. Furthermore, oil recovery yields may be increased because less amount of oil will be wasted to the aqueous phase.

Further, an advantage of the process described herein may be that an oil-mill using this process may skip sludge recycling of the polluted water used in the process.

The in the art known enzymatic degumming processes give rise to a high amount of polluted water, which is expensive to clean up. This is of course an economically burden.

Further oil-mills traditionally have been forced to implement recycling of the water processes in order to save cost in said purifying of the polluted water.

Said recycling step may be saved by the low amount of water used in the process described herein.

In enzymatic degumming carried out according to the art (e.g. US 5,264,367) a heat treatment to e.g. 65-75 °C of the water in oil emulsion is usually carried out in order to facilitate separation of the oil and aqueous phases by e.g. centrifugation. When using the thermostable phospholipase Lecitase™ (Novo Nordisk A/S, Denmark) in the oil degumming process, the aqueous phase containing the enzyme can advantageously be reused several times (with or without addition

of fresh enzyme solution).

However, for the oil mill it may be advantageous if the recycling of the aqueous phase could be totally omitted. This would in the normal case mean that overall water consumption would be increased with increased costs. If only a low amount of water is used in the enzymatic degumming process, recycling of the sometimes problematic sludge phase could be omitted.

Embodiment(s) of the present invention is described below, by way of example(s) only.

DETAILED DESCRIPTION OF THE INVENTION

Edible oils:

In principle any edible oil may be degummed according to a process of the invention. Example of oils are crude oils and water degummed oils.

A crude oil (also called a non-degummed oil) may be a pressed or extracted oil or a mixture thereof from e.g. rapeseed, soybean, or sunflower. The phosphatide content in a crude oil may vary from 0.5-3% w/w corresponding to a phosphorus content in the range of 200-10.000 ppm, more preferably in the range of 250-1200 ppm. Apart from the phosphatides the crude oil also contains small concentrations of carbohydrates, sugar compounds and metal/phosphatide acid complexes of Ca, Mg and Fe.

Preferably, said edible oil is an oil from which mucilage has previously been removed and which has a phosphorus content from 50 to 250 ppm.

Such an oil is generally obtained by a water-degumming process and termed "a water-degummed oil".

A water-degummed oil is typically obtained by mixing 1-3% w/w of hot water with warm (60-90°C) crude oil. Usual treatment periods are 30-60 minutes. The water-degumming step removes the phosphatides and mucilaginous gums which become insoluble in the oil when hydrated. The hydrated phosphatides and gums can be separated from the oil by settling, filtering or centrifuging - centrifuging being the more prevalent practice.

Alternatively, the process here termed "water-degumming" may be called "wet refining to remove mucilage" (see US 5,264,367).

Further, an edible is preferably an vegetable oil.

A Phospholipase used in the process:

Preferably, a phospholipase used in the process of the invention is a phospholipase obtained from a microorganism, preferably a filamentous fungus, a yeast, or a bacterium.

For the purpose of the present invention the term "obtained from", as used herein in connection with a specific microbial source, means that the enzyme and consequently the DNA sequence encoding said enzyme is produced by the specific source.

The enzyme is then obtained from said specific source by standard known methods enabling the skilled person to obtain a sample comprising the enzyme and capable of being used in a process of the invention. Said standard methods may be direct purification from said specific source or cloning of a DNA sequence encoding the enzyme followed by recombinant expression either in the same source (homologous recombinant expression) or in a different source (heterologous recombinant expression).

More preferably, a phospholipase used in a process of the invention is obtained from a filamentous fungal species within the genus *Fusarium*, such as a strain of *F. culmorum*, F. *heterosporum*, *F. solani*, or in particular a strain of *F. oxysporum*; or

a filamentous fungal species within the genus *Aspergillus*, such as a strain of *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus niger* or in particular *Aspergillus oryzae*.

Examples of suitable *Fusarium* phospholipases are disclosed in

i) Tsung-Che et al. (Phytopathological notes 58:1437-38 (1968)) (a phospholipase from *Fusarium solani)*; and

ii) EP Patent Application No. 97610056.0 disclosing a suitable *F. culmorum PL* (see example 18 in said doc.) and a suitable *F. oxysporum PL* (see example 1-17).

Suitable *Aspergillus* phospholipases are diclosed in

i) EP 575133 disclosing numerous different Aspergillus PL`s (see claim 14) and in particular a PL from A. oryzae(Claim

17 or 18) and a PL from A. niger (claim 19); and

ii) DE 19527274 A1 dicloses a suitable Aspergillus preparation (see examples).

Further the commercial available phospholipase preparation Degomma VOD (Roehm, Germany), which is believed to comprise an Aspergillus phospholipase is suitable to be used in a process of the invention.

Further, it is preferred that a phospholipase used in a process of the invention exhibits certain properties.

Accordingly, embodiment of the invention relates to

i) a process according to the invention, wherein the phospholipase is a phospholipase which is substantively independent of Ca^{2+} concentration measured as,

relative phospholipase activity at 5 mM EDTA and 5mM Ca^{2+} in a phospholipase activity assay measuring release of free fatty acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min.;
wherein the ratio of relative phospholipase activity at 5mM EDTA/5 mM Ca^{2+} is greater than 0.25, more preferably greater than 0.5; and/or

ii) a process according to the invention, wherein the phospholipase is a phospholipase which has a phospholipase activity which is capable of releasing at least 7 µmol of free fatty acid/min./mg enzyme; more preferably at least 15 µmol of free fatty acid/min./mg enzyme; measured as,

phospholipase activity is measured in an assay measuring release of free fatty acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min..

A detailed description of above mentioned assays is disclosed in a working example herein (*vide infra*). For even further details reference is made to EP Patent Application No. 97610056.0 (see example 9 in said document).

Further it has been demonstrated that a phospholipase special suited for enzymatic oil degumming in general and in

particular for the improved process described herein is characterized by having a certain primary amino acid sequence.

Accordingly, in an even further embodiment the invention relates to a process according to the invention, wherein the phospholipase is a phospholipase having an polypeptide sequence selected from the group comprising of:

(a) polypeptide having an amino acid sequence as shown in positions 31-346 of SEQ ID NO 1;

(b) a polypeptide having an amino acid sequence as shown in position 31-303 of SEQ ID NO 1;

(c) a polypeptide which is at least 70 % homologous with said polypeptide defined in (a), or (b); and

a fragment of (a), (b) or (c).

For a detailed description of cloning and purification of a phospholipase having the above mentioned polypeptide sequence reference is made to EP Patent Application No. 97610056.0.

In this document it can further be seen that a phospholipase obtained from *F. oxysporum* and having the polypeptide sequence shown in (b) above exhibits both of the above mentioned functional characteristic. Accordingly, this phospholipase is the most preferred phospholipase to be used in a process of the invention. A working example herein demonstrates the use of this phospholipase (vide infra).

Finally an example of a suitable non-microbial phospholipase is the commercial available PL (Lecitase™, Novo Nordisk A/S, Denmark) obtained from porcine pancreas.

<u>Standard process parameters of the process of the invention:</u>

Besides the specific use of low amount of water in the process of the invention, any of the other process parameters may be done according to the art. See Background section above for references to the art known processes.

The enzymatic treatment is conducted by dispersing an aqueous solution of the phospholipase, preferably as droplets with an average diameter below 10 µ(micro)m.

According to the process of the invention the amount of water is from 0.01 to 1.5% by weight in relation to the oil.

An emulsifier may optionally be added. Mechanical agitation may be applied to maintain the emulsion.

The enzymatic treatment can be conducted at any pH in the range 1.5-8, preferably from pH 3-6. The pH may be adjusted by adding citric acid, a citrate buffer, NaOH or HCl.

A suitable temperature is generally 30-75°C (particularly 40-60°C). The reaction time will typically be 0.5-12 hours (e.g. 2-6 hours), and a suitable enzyme dosage will usually be 100-5000 IU per liter of oil, particularly 200-2000 IU/l.

The enzymatic treatment may be conducted batchwise, e.g. in a tank with stirring, or it may be continuous, e.g. a series of stirred tank reactors.

The enzymatic treatment is followed by separation of an aqueous phase and an oil phase. This separation may be performed by conventional means, e.g. centrifugation. The process of the invention can reduce this value to below 12 ppm, more preferably below 10, and even more preferably below 5 ppm.

MATERIALS AND METHODS

EXAMPLES

EXAMPLE 1

General description of assay for enzymatic degumming of edible oil

Equipment for carrying out enzymatic degumming

The equipment consists of a 1 l jacketed steel reactor fitted with a steel lid, a propeller (about 600 rpm), baffles, a temperature sensor, an inlet tube at the top, a reflux condenser (about 4°C) at the top, and an outlet tube at the bottom. The reactor jacket is connected to a thermostat bath. The outlet tube is connected via silicone tubing to a Silverson in-line mixer head equipped with a "square hole high shear screen", driven by a Silverson L4RT high shear lab mixer (about 8500 rpm, flow ca. 1.1 l/minute). The mixer head is fitted with a cooling coil (5-10 °C) and an outlet tube, which is connected to the

inlet tube of the reactor via silicone tubing. A temperature sensor is inserted in the silicone tubing just after the mixer head. The only connection from the reactor/mixer head system to the atmosphere is through the reflux condenser.

General procedure for carrying out enzymatic degumming

All cooling and thermostat equipment is turned on. Then 0.6 l (ca. 560 g) of oil is loaded in the reactor, which is kept at about the temperature needed for the specific experiment. The lab mixer is turned on, whereby the oil starts to circulate from the reactor to the mixer head and back to the reactor. The system is allowed to equilibrate for about 10 minutes, during which period the temperature is fine tuned. The pre-treatment period starts with addition of 0.6 g (2.86 mmol) citric acid monohydrate in the appropriate amount of water or the appropriate amount of a mixture of citric acid and trisodium citrate (see Tables 1 and 7 below; [citric acid] in water/oil emulsion = 4.6 mM), which sets t = 0. At t = 30 minutes the appropriate amount of 4 M NaOH solution is added (see Tables 1 and 7).

Table 1. Water content in Experiments A-D; wdg rape seed oil.

Experiment	Water content	Water in 560 g oil	Water added at t=0	Water in NaOH solution	Water in enzyme solution	Total water
A	5.3 %	0.56 g	27 g	1.1 g	1.0 g	29.7 g
B	1.3 %	0.56 g	5.0 g	0.7 g	1.0 g	7.3 g
C	0.3 %	0.56 g	0.05 g*	0 g	1.0 g	1.6 g
D	0.3 %	0.56 g	0.07 g**	0 g	1.0 g	1.6 g

* Water contribution from o.6 g citric acid monohydrate.

** Water contribution from mixt. of 0.5 g citric acid monohydrate and 0.14 g trisodium citrate dihydrate.

At t = 35 minutes samples are drawn for P-analysis and pH determination. Just after this the required amount of enzyme

solution is added (end of pre-treatment period). Samples for P-analysis and pH determination are drawn at t = 1, 2, 3.5, 5, 6 hours, and then the reaction is stopped.

The reactor/mixer system is emptied and rinsed with 2×500 ml 10% Deconex/DI water solution followed by minimum 3×500 ml of DI water. Table 2 is a presentation of the various additions and samplings during the reaction.

Table 2. Schedule for enzymatic degumming

Time	Addition of	Sampling	
		P-analysis	pH determination
		X	
0	Citric acid		
5 min.			X
30 min.		X	X
30 + δ min.	NaOH		
35 min.		X	X
35 + δ min.	Enzyme		
1 hour		X	X
2 hours		X	X
3.5 hours		X	X
5 hours		X	X
6 hours		X	X

Phosphorus analysis:

Sampling for P-analysis:

Take 10 ml of water in oil emulsion in a glass centrifuge tube. Heat the emulsion in a boiling water bath for 30 minutes. Centrifuge at 5000 rpm for 10 minutes. Transfer about 8 ml of upper (oil) phase to a 12 ml polystyrene tube and leave it (to settle) for 12-24 hours. After settling draw about 1-2 g from the upper clear phase for P-analysis.

P-analysis was carried out according to procedure 2.421 in

"Standard Methods for the Analysis of Oils, Fats, and Derivatives, 7th ed. (1987)":

Weigh 100 mg of MgO (leicht, Merck #5862) in a porcelain dish and heat with a gas burner. Add 1-2 g of oil and ignite with a gas burner to give a black, hard mass. Heat in a Vecstar furnace at 850°C for 2 hours to give white ashes. Dissolve the ashes in 5 ml of 6 M HNO_3 and add 20 ml of reagent mix. Leave for 20 minutes. Measure absorbance at 460 nm (use a blank (5 ml HNO_3 + 20 ml reagent mix) for zero adjustment). Calculate by using calibration curve.

pH determination

Take 2 ml of water in oil emulsion and mix with 2 ml of MilliQ water. After phase separation, pipette off top oil layer. Measure pH in aqueous phase with pH electrode Orion. Measurements are transformed to "real" pH values by the formula

$$pH_{real} = pH_{measured} - 0.38.$$

A calibration curve was obtained by dissolving 0.6 g of citric acid monohydrate in 27 g of DI water; pH of this solution was measured by pH electrode Orion (pH_{real}). 100 µl were mixed with 2 ml MilliQ water, and pH of this solution was measured by pH electrode Orion ($pH_{measured}$). pH of the citric acid solution was changed gradually by adding NaOH solution, and for each adjustment dilution and pH measurements were carried out as described above.)

EXAMPLE 2

Degumming of water-degummed rape seed oil (I)

Experiments were carried out according to the "General procedure for carrying out enzymatic degumming" as described in example 1 above.

Oil:

Water-degummed rape seed oil from Århus Oliefabrik (AOM), Denmark. Batches C00730/B01700 and C00730/B01702, P-content 231-236 ppm. Water content ≤ 0.1 % w/w.

Enzyme:

PL from *Fusarium oxysporum* having the amino acid sequence shown in SEQ NO 1.

Batch F-9702027, estimated conc. 0.75 mg/ml.

The enzyme was recombinantly expressed and purified as described in EP Patent application number 97610056.0.

Experiment A (water content 5.3 %)

0.6 l (560 g) of wdg rape seed oil is loaded in the equipment and heated to 40°C. At t = 0 min. a solution of 0.6 g of citric acid monohydrate in 27 g of water was added. At t = 30 min. 1.07 ml (4.3 mmoles) of 4 M NaOH solution were added, which yield a pH of about 5. At t = 35 min., 1 ml (0.75 mg) of a purified solution of phospholipase from *F. oxysporum* is added. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 3.

Table 3. Results from degumming of wdg rape seed oil with phospholipase from *F. oxysporum*, water content 5.3 %.

Time (hours)	Phosphorus content in oil phase	pH
0	243	
0.50	215	4.7
0.58	216	5.5
1.0	66	4.9
2.0	10	4.9
3.5	8	5.4
5.0	9	5.0

Experiment B (water content 1.3 %)

As in Experiment A above except that at t = 0 min. 0.6 g of

citric acid monohydrate in 5.0 g of water was added, and at t = 30 min. 0.71 ml (2.86 mmoles) of 4 M NaOH solution were added which yield a pH of about 5. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 4.

Table 4. Results from degumming of wdg rape seed oil with phospholipase from *F. oxysporum*, water content 1.3 %.

Time (hours)	Phosphorus content in oil phase	pH
0	237	
0.50	213	4.7
0.58	197	5.7
1.0	78	4.9
2.0	9	4.9
3.5	10	5.0
5.0	12	5.1
6.0	10	5.0

Experiment C (water content 0.3 %)

As in Experiment A above except that at t = 0 min. 0.6 g of citric acid monohydrate powder was added, and at t = 30 min. no NaOH solution was added, which yield a pH of about 5. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 5.

Table 5. Results from degumming of wdg rape seed oil with phospholipase from *F. oxysporum*, water content 0.3 %.

Time (hours)	Phosphorus content in oil phase	pH
0	246	4.9
0.50	234	5.1
0.58		
1.0	101	4.8
2.0	18	5.2
3.5	11	5.2

Experiment D (water content 0.3 %)

As in Experiment C above except that at t = 0 min. a mixture of 0.5 g of citric acid monohydrate and 0.14 g trisodium citrate dihydrate powder was added, which yield a pH of about 5. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 6.

Table 6. Results from degumming of wdg rape seed oil with phospholipase from *F. oxysporum*, water content 0.3 %.

Time (hours)	Phosphorus content in oil phase	pH
0	243	
0.50	244	5.5
0.58		
1.0	101	5.1
2.0	8	4.9

EXAMPLE 3

Degumming of crude (mixture of pressed and extracted) rape seed oil (II)

Experiments were carried out according to the "General procedure for carrying out enzymatic degumming" as described in example 1 above.

Oil:

Crude rape seed oil from MILO Olomouk, Czech rep. Batch C00745/B02042, P-content 263 ppm. Water content 0.17 % w/w.

Table 7. Water content in Experiments E and F; crude rape seed oil.

Experiment	Water content	Water in 560 g oil	Water added at t=0	Water in NaOH solution	Water in enzyme solution	Total water
E	5.4 %	0.95 g	27 g	1.1 g	1.0 g	30.1 g
F	1.4 %	0.95 g	5.0 g	0.7 g	1.0 g	7.7 g

Experiment E (water content 5.4 %)

0.6 l (560 g) of crude rape seed oil is loaded in the equipment and heated to 40°C. At t = 0 min. a solution of 0.6 g of citric acid monohydrate in 27 g of water was added. At t = 30 min. 1.07 ml (4.3 mmoles) of 4 M NaOH solution were added, which yield a pH of about 5. At t = 35 min., 1 ml (0.75 mg) of a purified solution of phospholipase from *F. oxysporum* is added. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 8.

Table 8. Results from degumming of crude rape seed oil with phospholipase from *F. oxysporum*, water content 5.4 %.

Time (hours)	Phosphorus content in oil phase	pH
0	222	
0.50	165	
0.58	136	4.8
1.0	38	5.1
2.0	10	5.0
3.5	11	5.0
5.0	11	5.0
6.0	10	5.3

Experiment F (water content 1.4 %)

As in Experiment E above except that at t = 0 min. 0.6 g of citric acid monohydrate in 5.0 g of water was added, and at t = 30 min. 0.71 ml (2.86 mmoles) of 4 M NaOH solution were added which yield a pH of about 5. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 9.

Table 9. Results from degumming of crude rape seed oil with phospholipase from *F. oxysporum*, water content 1.4 %.

Time (hours)	Phosphorus content in oil phase	pH
0	223	
0.50	119	
0.58	92	5.1
1.0	31	5.1
2.0	12	5.0
3.5	11	5.1
5.0	9	4.8
6.0	8	4.3

EXAMPLE 4

Assays used for characterization of a phospholipase suitable to be used in an oil degumming process of the invention.

Phospholipase activity assays:

Phospholipase activity (PHLU) was measured as the release of free fatty acids from lecithin. 50 µl 4% L-alpha-phosphatidylcholine (plant lecithin from Avanti, USA), 4% Triton X-100, 5 mM $CaCl_2$ in 50 mM HEPES, pH 7 was added, 50 µl enzyme solution diluted to an appropriate concentration in 50 mM HEPES, pH 7. The samples were incubated for 10 min at 30°C and the reaction stopped at 95°C for 5 min prior to centrifugation (5 min at 7000 rpm). Free fatty acids were determined using the NEFA C kit from Wako Chemicals GmbH; 25 µl reaction mixture was added to 250 µl reagent A and incubated for 10 min at 37°C. Then 500 µl Reagent B was added and the sample was incubated again, 10 min at 37°C. The absorption at 550 nm was measured using an HP 8452A diode array spectrophotometer. Samples were run at least in duplicates. Substrate and enzyme blinds (preheated enzyme samples (10 min at 95°C) + substrate) were included. Oleic acid was used as a fatty acid standard. 1 PHLU equals the amount of enzyme capable of releasing 1 µmol of free fatty acid/min under these conditions.

Alternatively, the assay was run at 37°C in 20 mM citrate buffer, pH 5 (Ca^{2+}-dependence) or 20 mM Britton-Robinson buffer (pH-profile/temperature-profile/stability).

Phospholipase A1 activity (PLA1) was measured using 1-(S-decanoyl)-2-decanoyl-1-thio-sn-glycero-3-phosphocholine (D3761 Molecular Probes) as a substrate. 190 µl substrate (100 µl D3761 (2 mg/ml in ethanol) + 50 µl 1 % Triton X-100 + 1.85 ml 50 mM HEPES, 0.3 mM DTNB, 2 mM $CaCl_2$, pH 7) in a 200 µl cuvette were added to 10 µl enzyme, and the absorption at 410 nm was measured as a function of time on the HP 8452A diode array spectrophotometer at room temperature. Activity was calculated as the slope of the curve in the linear range. PLA1 equals the amount of enzyme capable of releasing 1 µmol of free fatty acid (thiol)/min at these conditions.

Phospholipase A2 activity (PLA2) was measured at 40°C using 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (H361 Molecular Probes). 2 ml substrate (50 µl 1% Triton X-100 + 25 µl 0.1% H361 in methanol + 10 ml 50mM HEPES, pH 7) in a 2 ml cuvette with stirring was added to 10 µl enzyme, and the pyrene fluorescence emission was measured at 376 nm (excitation at 340 nm) as a function of time (1 sec. intervals) using the Perkin Elmer LS50 apparatus. In the Triton X-100/phospholipid micelles the concentration of phospholipid was adjusted to have excimer formation (emits at 480 nm). Upon cleavage the fatty acid in the 2-position containing the pyrene group is released into the aqueous phase resulting in an increase in the monomer emission. PLA2 was taken as the slope of the curve in the linear range at equal conditions.

CLAIMS

1. A process for reducing the content of phosphorus containing components in an edible oil, having from 50 to 10.000 part per million (ppm) of phosphorus content, which method comprises contacting said oil at a pH from 1.5 to 8 with an aqueous solution of a phospholipase A1 (PLA1), phospholipase A2 (PLA2), or phospholipase B (PLB) which is emulsified in the oil until the phosphorus content of the oil is reduced to less than 12 ppm, and then separating the aqueous phase from the treated oil, and wherein said process is characterized by that said emulsified condition is formed using from 0.01 to 1.5 percent of water by weight of the oil, preferably from 0.01 to 1.0 percent of water by weight of the oil, and most preferably from 0.01 to 0.5 percent of water by weight of the oil.

2. The process according to claim 1, wherein said oil is an oil from which mucilage has previously been removed and which has a phosphorus content from 50 to 250 ppm.

3. The process according to claims 1 or 2, wherein the phospholipase is an phospholipase obtained from a microorganism, preferably a filamentous fungus, a yeast, or a bacterium.

4. The process according to claim 3, wherein the filamentous fungus is a species within the genus *Fusarium*, such as a strain of *F. culmorum*, F. *heterosporum*, *F. solani*, or in particular a strain of *F. oxysporum*.

5. The process according to claim 3, wherein the filamentous fungus is a species within the genus *Aspergillus*, such as a strain of *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus niger* or in particular *Aspergillus oryzae*.

6. The process according to any of the preceeding claims, wherein the phospholipase is a phospholipase which is substantively independent of Ca^{2+} concentration measured as, relative phospholipase activity at 5 mM EDTA and 5mM Ca^{2+} in a phospholipase activity assay measuring release of free fatty

acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min.;
wherein the ratio of relative phospholipase activity at 5mM EDTA/5 mM Ca^{2+} is greater than 0.25, more preferably greater than 0.5.

7. The process according to any of the preceding claims, wherein the phospholipase is a phospholipase which has a phospholipase activity which is capable of releasing at least 7 µmol of free fatty acid/min./mg enzyme; more preferably at least 15 µmol of free fatty acid/min./mg enzyme; measured as,
phospholipase activity is measured in an assay measuring release of free fatty acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min..

8. The process according to any of the preceding claims, wherein the phospholipase is a phospholipase having an polypeptide sequence selected from the group comprising of:
(a) polypeptide having an amino acid sequence as shown in positions 31-346 of SEQ ID NO 1;
(b) a polypeptide having an amino acid sequence as shown in position 31-303 of SEQ ID NO 1;
(c) a polypeptide which is at least 70 % homologous with said polypeptide defined in (a), or (b); and
a fragment of (a), (b) or (c).

SEQUENCE LISTING

<110> NOVO NORDISK A/S

<120> AN ENZYMATIC OIL-DEGUMMING PROCESS

<130> 5570-WO

<140>
<141>

<160> 1

<170> PatentIn Ver. 2.0

<210> 1
<211> 346
<212> PRT
<213> Fusarium oxysporum

<400> 1

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Gly Val Thr Thr Thr Asp Phe Ser Asn Phe Lys Phe Tyr Ile Gln His
         35                  40                  45

Gly Ala Ala Ala Tyr Cys Asn Ser Glu Ala Ala Ala Gly Ser Lys Ile
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Thr Cys Ser Asn Asn Gly Cys Pro Thr Val Gln Gly Asn Gly Ala Thr
 65                  70                  75                  80

Ile Val Thr Ser Phe Val Gly Ser Lys Thr Gly Ile Gly Gly Tyr Val
                 85                  90                  95

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Ile Asn Ile Arg Asn Trp Leu Thr Asn Leu Asp Phe Gly Gln Glu Asp
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Cys Ser Leu Val Ser Gly Cys Gly Val His Ser Gly Phe Gln Arg Ala
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Pro Val Asp Ile Tyr Thr Tyr Gly Ser Pro Arg Val Gly Asn Ala Gln
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His Ala Asp Asp Pro Val Pro Arg Leu Pro Pro Leu Ile Phe Gly Tyr
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Arg His Thr Thr Pro Glu Phe Trp Leu Ser Gly Gly Gly Gly Asp Lys
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Val Asp Tyr Thr Ile Ser Asp Val Lys Val Cys Glu Gly Ala Ala Asn
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    290                 295                 300

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Asp Ala Glu Leu Glu Lys Lys Leu Asn Ser Tyr Val Gln Met Asp Lys
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Glu Tyr Val Lys Asn Asn Gln Ala Arg Ser
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00202

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C11B 3/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C11B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9826057 A1 (NOVO NORDISK A/S), 18 June 1998 (18.06.98), See sequence page 17, line 14-15 --	1-8
P,X	WO 9818912 A1 (NOVO NORDISK A/S), 7 May 1998 (07.05.98), See page 8, line 25, claim 27 --	1-8
X	File WPI, Derwent accession no. 90-226962, Showa Sangyo Co: "Purificn. of fat and oil, requiring no acid-removing process - by treating with enzyme having phospho-lipase A activity", JP,A,2153997, 900613, DW9030 --	1-8

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 July 1999	17 -07- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Yvonne Siösteen/Els Telephone No. + 46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00202

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	File WPI, Derwent accession no. 90-096521, Showa Sangyo Co: "Lysolecithin prepn. - by adding enzyme showing phospholipase A activity to oil", JP,A,2049593, 900219, DW9013	1-8
X	US 5264367 A (ERIK AALRUST ET AL), 23 November 1993 (23.11.93), See column 3, line 3	1-8
A	EP 0622446 A2 (SHOWA SANGYO CO., LTD.), 2 November 1994 (02.11.94), See page 3, lines 33-34, claim 4	1-8
A	US 5558781 A (HENNING BUCHOLD ET AL), 24 Sept 1996 (24.09.96)	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/06/99

International application No.
PCT/DK 99/00202

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		CN 1066679 A	02/12/92
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		DE 59201753 D	00/00/00
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		SE 0513709 T3	
		ES 2072043 T	01/07/95
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US 5558781 A	24/09/96	AT 162210 T	15/01/98
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